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***In Vivo* and *In Vitro* Bisphenol A Exposure Effects on Adiposity**

Mina Desai^{1,2}, Monica G. Ferrini³, Juanita K. Jellyman¹, Guang Han¹, and Michael G. Ross^{1,2,4}

¹Perinatal Research Laboratory, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Department of Obstetrics and Gynecology, Torrance, CA.

²Department of Obstetrics and Gynecology, David Geffen School of Medicine, University of California, Los Angeles, CA.

³Department of Health and Life Sciences Department of Internal Medicine, Charles R. Drew University, Los Angeles, CA

⁴Department of Obstetrics and Gynaecology, Charles R. Drew University, Los Angeles, CA

Abstract

In utero exposure to the ubiquitous plasticizer, bisphenol A (BPA) is associated with offspring obesity. As adipogenesis is a critical factor contributing to obesity, we determined the effects of *in vivo* maternal BPA and *in vitro* BPA exposure on newborn adipose tissue at the stem cell level. For *in vivo* studies, female rats received BPA prior to and during pregnancy and lactation via drinking water, and offspring were studied for measures of adiposity signals. For *in vitro* BPA exposure, primary preadipocyte cell cultures from healthy newborns were utilized. We studied preadipocyte proliferative and differentiation effects of BPA and explored putative signal factors which partly explain adipose responses, and underlying epigenetic mechanisms mediated by BPA. Maternal BPA induced offspring adiposity, hypertrophic adipocytes and increased adipose tissue protein expression of pro-adipogenic and lipogenic factors. Consistent with *in vivo* data, *in vitro* BPA exposure induced dose-dependent increase in preadipocyte proliferation and increased adipocyte lipid content. *In vivo* and *in vitro* BPA exposure promotes proliferation and differentiation of adipocytes, contributing to enhanced capacity for lipid storage. These findings reinforce the marked effects of BPA on adipogenesis and highlight the susceptibility of stem-cell populations during early life with long-term consequence on metabolic homeostasis.

Corresponding Author: Mina Desai, PhD, LABioMed at Harbor-UCLA Medical Center, 1124 West Carson Street Box 446, Liu Research Bldg., Torrance CA 90502, USA, Phone No: (310) 974 9540, FAX No: (310) 972 2962, mdesai@labiomed.org.

CONFLICTS OF INTEREST: None

ETHICAL STANDARDS: As stated in the Methods, all studies were approved by the Animal Research Committee of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center and were conducted in strict accordance with guidelines provided by the American Accreditation Association of Laboratory Care and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and conform to the principles and regulations as described in the Editorial by Grundy.⁸⁹ All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague Dawley female rats (Charles River Laboratories, Hollister, CA) were housed in an animal facility with controlled 12/12 hour light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood, Missouri) and water.

Keywords

Bisphenol A; Adipogenesis; Lipogenesis; PPARgamma; Proliferation; Differentiation

INTRODUCTION

The dramatic world-wide increase in obesity and its associated metabolic diseases has been widely attributed to an obesogenic environment created by our increasingly sedentary lifestyles and easy access to highly palatable, energy dense foods. While there is little doubt that poor diet and lack of exercise contribute to the obesity epidemic, recent studies have identified an estrogen endocrine disrupter chemical Bisphenol A (BPA) that may act as an environmental obesogen and either directly, or indirectly influence fat accrual.¹ BPA, a monomer plasticizer, is used in the manufacture of common house-hold goods including polycarbonate plastics (e.g. food and drink containers), paints and adhesives (Vandenberg et al. 2007). In rodents, maternal BPA exposure increases postnatal body weights and growth rates with differing sex-specific effects. Some studies show greater susceptibility to BPA-increased adiposity in females, some in males and other studies show effects on both sexes.²⁻⁶ Critically, fetal exposures to BPA at levels equivalent to, or below the established daily human safe-dose (50µg BPA/kg body weight/day) not only increase body weight and postnatal growth rate, but also alter body composition in later life.^{2, 3, 7-9} Energy (calorie) intake, energy expenditure and energy storage are the three key determinants of energy balance.¹⁰ Increased adipose mass in offspring exposed to BPA during pregnancy may result from increased numbers of adipocyte cells (hyperplasia) secondary to enhanced differentiation of pre-adipocytes into mature adipocyte cells, as well as increased adipocyte cell size (hypertrophy) secondary to triglyceride storage.^{11,12} Adipogenesis involves preadipocyte proliferation, differentiation and lipogenesis.^{13,14} This process requires the coordinated interaction of preadipocyte proliferative factors (Pref-1, SOX9) and several adipogenic differentiation transcription factors, including members of the CCAAT/ enhancer binding family of proteins (C/EBPα, C/EBPβ and C/EBPδ),^{15,16} which activate the peroxisome proliferator-activated receptor (PPARγ₂).¹⁷ BPA is associated with increased expression of pro-adipogenic genes *in vivo*^{4,18} and with accelerated terminal differentiation of 3T3-L1 cells *in vitro*^{19,20}. However, no study to date has determined the effect of prenatal BPA on offspring primary pre-adipocyte proliferation and differentiation, and the underlying mechanism.

We sought to confirm the effects of maternal BPA exposure during pregnancy and lactation on offspring body weight, while examining effects on measures of adiposity. To more fully explore the mechanisms of BPA-mediated effects, we further utilized established models of newborn rat primary pre-adipocyte stem cells, exploring both proliferative (i.e., trophic) and differentiation effects of BPA.²¹ We further explored putative signal factors which explain, in part, adipose responses, and underlying epigenetic mechanisms mediated by BPA. The data reinforce the marked effects of BPA on adipogenesis and highlight the susceptibility of stem-cell populations during early life with long-term consequence on metabolic homeostasis.

METHODS

BPA Model

In Vivo Maternal BPA Exposure: Studies were approved by the Animal Care Committee at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center and were in accordance with the American Accreditation Association of Laboratory Care. All animals were treated humanely and with regard for alleviation of suffering. Virgin Sprague Dawley female rats (Charles River Laboratories, Hollister, CA) were housed in an animal facility with controlled 12/12 hour light/dark cycles, constant temperature and humidity conditions and *ad libitum* access to chow diet (Lab Diet 5001; Brentwood, Missouri). The chow diet contains soy meal and as this was fed to both Controls and BPA exposed animals, any comparative differences between the groups are likely due to the BPA exposure rather than the estrogenic activity of the phytoestrogens in the diet. To avoid potential BPA contamination, polypropylene cages and purified water in glass bottles were utilized. Female rats were randomly assigned to Control (n=5) or BPA (n=5) group. To reflect the most likely route of human exposure,^{22–25} dams were exposed to BPA via their drinking water. Control rats had access to purified drinking water, whereas the BPA group received purified drinking water containing BPA (5mg/L; BPA Sigma-Aldrich, purity 99%, CAS no. 80–05-7) for two weeks prior to mating and throughout pregnancy and lactation (Table 1). Studies that administered BPA to pregnant rodents via drinking water, a concentration of 10 mg/l water (consumption of ~1.2 mg/kg BW/day)²⁶ yielded BPA tissue concentrations of 10–25 ng/g tissue^{27,28} consistent with that of human samples.²⁹ A dose five-fold higher (6 mg/kg BW/day) administered via gavage, achieved significantly higher maternal plasma BPA levels,³⁰ whereas a water concentration of only 1 mg/l resulted in low maternal plasma free BPA levels (0.84 ng/ml).³¹ Our dose was selected based upon our confirmation (pilot study) of maternal and newborn serum levels within the lower range of demonstrated human levels with normal BPA exposure.

Maternal body weight and water intake was monitored daily, and DEXA was undertaken at end of lactation after which adipose tissue was collected for cell size analysis. Prior to mating, maternal blood was obtained via tail bleed and at end of lactation via cardiac puncture in BPA-free tubes for BPA analysis. To avoid inducing maternal stress and fetal resorption,³² blood samples were not collected during pregnancy, especially as maternal stress has been demonstrated to be an independent risk factor for offspring obesity.^{33,34} Free (unconjugated) BPA levels were measured using GC/MS (NMS Labs, PA) with assay sensitivity of 0.25 ng/ml. Insufficient plasma volume from maternal tail bleed (prior to BPA administration) and newborns necessitated pooling of samples and hence only mean values are reported. Following BPA administration at end of lactation, samples were analyzed individually for BPA levels.

Dams gave birth spontaneously and 5 litters per group (Control and BPA) were utilized for offspring studies. Litter size was standardized to eight per litter (4 males and 4 females) to normalize rearing and all offspring were nursed by their respective mothers till 3 weeks of age. Following weaning, all offspring were given purified water and housed in polypropylene cages.

Offspring Studies

DEXA Scan: At 3 and 24 weeks of age, one male and one female offspring from one litter (N=5) underwent a non-invasive dual-energy x-ray absorptiometry (DEXA) scanning using DXA system with software program for small animal (QDR 4500A, Hologic, Bedford, MA, USA). An *in vivo* scan of whole body composition was obtained, including fat tissue mass, total mass and percent body fat.

Adipose Tissue Retrieval: Excess pups (beyond the 4 males and 4 females) were sacrificed at day one of life and subcutaneous (inguinal) adipose tissue from two pups of each gender from each litter was pooled according to sex (representing N=1) for analysis of tissue protein expression. A total of N=5 litters were studied. At 3 weeks of age, one male and one female from one litter (N=5) were euthanized and visceral (retroperitoneal) adipose tissue was collected for analysis of protein expression.

Blood Pressure: At 6 weeks of age, measurements were undertaken in conscious animals using non-invasive tail-cuff sphygmomanometry (ML125 NIPB System, AD Instruments) method. Several cuff sizes are used depending on the weight of the animal. To circumvent the potential problem of restraint-induced stress, the animals were acclimatized for at least one week with placement in the restraint. One male and one female offspring from one litter (N=5) were studied.

In Vitro BPA Exposure: An additional four Control litters (N=4) were studied for *in vitro* effects of exogenous BPA on adipocyte cultures. From each litter, inguinal adipose tissue was dissected from each of four, 1 day old Control males (four pooled samples representing N=1) for cell culture studies. Preadipocytes were cultured in DMEM medium in absence or presence of differentiation cocktail (see below) and treated with DMSO (control) or BPA (1, 10, 20 μ M) for 5 days. The total number studied was N=4 from 4 litters for adipocyte cultures.

Primary Cultures

Primary Adipocyte Cell Cultures—were established as previously described.²¹ Briefly, pooled adipose tissue from 1 day old newborn males was minced and digested with collagenase type II (5000 U/g) in Krebs-Ringer solution. The preadipocyte cultures were resuspended in high glucose (450 mg/dl) Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotics (Invitrogen) and incubated at 37 °C with 5% CO₂. Cells were either seeded in 24-well plate ($\sim 1 \times 10^4$ cells/ml) for proliferation studies or flasks ($\sim 1 \times 10^6$ cells/ml) for protein expression and differentiation studies. After 24h, undifferentiated preadipocytes were either treated with DMSO or BPA for 5 days. For differentiation studies, 24h cultured preadipocytes were treated with dexamethasone (1 μ M), methylisobutylxanthine (0.1 mM), insulin (10 μ g/ml) in presence of DMSO or BPA for 5 days. At end of exposure, all cells were harvested and protein extracted for analysis as described under Western Blot.

Analysis

Pre-adipocyte Proliferation Assay: Pre-adipocyte (complete medium) proliferation was determined as previously reported²¹ using MTT colorimetric assay.³⁵

Adipocyte Cell Size: Cross-sectional area of adipocytes was determined as previously described.³⁶ Briefly, formalin-fixed and paraffin-embedded adipose tissue was sectioned (5µm), stained with hematoxylin-eosin, photomicrographs captured at 20× magnification and area of adipocytes (3 images per section, and 3 sections per animal) determined using Image PRO software.

Lipid Staining: Differentiated adipocytes were fixed with 4% paraformaldehyde, stained with 0.5% oil red O, mounted onto slides with Vectashield mounting medium with 4',6-diamidino-2 phenylindole (DAPI; Vector) and images (40X magnification) captured (Zeiss Axioskop 40 microscope with Axiocam HRc camera). For quantification, stained adipocytes were dried for 1 hour at 37°C, incubated with a fixed volume of isopropanol for 20 minutes to elute the oil red O, and absorbance measured at 520 nm.

Western Blot: Cell and tissue protein was extracted and Western Blotting performed as described previously.³⁶ For adipose tissue, protein expression analysis included preadipocyte marker (Pref-1, 55 kDa, Millipore); suppressor of preadipocyte differentiation (SOX9, Millipore); adipogenic markers (PPARγ, 57 kDa, Cayman; C/EBPα, 42 kDa, Santa Cruz); lipogenic factor (SREBP1, 125 kDa, Santa Cruz), and; epigenetic regulators DNA methyltransferase 3a (DNMT3a, 120 kDa, Santa Cruz) and lysine (K)-specific demethylase 1A (LSD1, 110 kDa, Cell Signaling).

Statistical Analyses:

In vivo responses between BPA and control offspring were compared by unpaired t-test. *In vitro* responses between BPA exposed and untreated (DMSO) Control cells were compared by unpaired t-test or analysis of variance (ANOVA) with Dunnett's post-hoc test, as appropriate. P values < 0.05 were considered significant.

RESULTS

Maternal BPA Effects on Maternal Phenotype:

Of note, the higher BPA levels (shown below) did not impact maternal weight gain and body fat. The maternal body weight and water intake during pregnancy and lactation was comparable between BPA and Control dams (Fig 1A). Further at end of lactation, body fat and adipocyte cell size were similar in both groups (Fig 1B).

Maternal BPA Effects on Plasma BPA Levels:

The pooled maternal plasma BPA level prior to BPA administration was 0.46 ng/ml. During the course of pregnancy, the amount of BPA consumed by dams via drinking water was 500–900 µg/kg/day and during lactation, it was higher (approximately 1500 µg/kg/day) due to increased water intake. The maternal BPA plasma levels at end of lactation were higher in BPA as compared to Control dams (8.2±3.8 vs. 0.42±0.04ng/ml). Similarly, newborns of

BPA dams had higher plasma BPA levels (0.62 ng/ml) as compared to undetectable levels in newborns of Control dams.

Maternal BPA Effects on Offspring Phenotype:

Maternal BPA did not alter birth weights of either male or female newborns. However by end of the nursing period, 3 week old BPA males exhibited significantly increased body weight that persisted in 24 week old adults (Fig 2). Consistent with this, the percentage of body fat was increased in 3 and 24 week old BPA males as compared to Controls (Fig 3). Additionally, the systolic blood pressure was significantly higher in 6 week old BPA than Control males (Fig 4). In contrast to males, body weights, percentage body fat and systolic blood pressure of BPA females were comparable to Control females (Figs 2, 3, 4).

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As adiposity was not altered in BPA female, we only studied male offspring. At 1 day of age, BPA males had significantly increased protein expression of the adipogenic transcription factor PPAR γ though not C/EBP α or the lipogenic factor SREBP1 (Fig 5a). At 3 weeks of age, BPA males showed increased protein expression of C/EBP α though not PPAR γ while the lipogenic factor SREBP1 was increased (Fig 5b) in conjunction with evidence of hypertrophic adipocytes (Figure 5c).

Consistent with increased adipose tissue mass and hypertrophic adipocytes in BPA males, protein expression of CD68 (macrophage marker) and TNF α (proinflammatory cytokine) was significantly increased at 3 weeks of age (Fig 6).

In Vitro BPA Effects

Pre-adipocytes from Control offspring cultured in standard media with incremental doses of BPA showed a dose-dependent increase in proliferation (Fig 7a, b), consistent with increased expression of the preadipocyte marker (Pref-1), though expression of the anti-adipocyte differentiation transcription factor (SOX9) expression was decreased at 10 μ M BPA (Fig 7c). In differentiated adipocytes (grown in DMEM medium), BPA increased the number of adipocytes (1.8-fold), consistent with the increased protein expression of adipogenic transcription factors (PPAR γ , C/EBP α), and expression of the proinflammatory cytokine TNF α (Fig 8a). In addition, BPA treated adipocytes showed increased lipid accumulation consistent with increased expression of SREBP1 (Fig 8b).

Epigenetic Factors: BPA treated adipocytes demonstrated a dose-dependent increase in DNMT3 though not LSD1 (Fig 9).

DISCUSSION

The present study determined the effects of BPA exposure *in vivo* and *in vitro* on adipogenesis, and the protein expression of regulatory transcription factors in early life. The data suggest that enhanced preadipocyte proliferation and differentiation in early life may contribute to the underlying mechanism of BPA-induced obesity.

A wide range of detectable BPA levels are reported in adults and children serum (0.2–20 ng/ml),³⁷ including breast milk (1.1 ng/ml), and maternal (0.2–10 ng/ml) and fetal/newborn serum (0.2–9.2 ng/ml). More significantly, the higher levels of measurable BPA in amniotic fluid (8.3–8.7 ng/ml) and placental tissues (1.0–104.9 ng/ml),^{38,39} imply a continued fetal exposure to BPA throughout development. Of note, higher BPA levels are seen in infants and children than in adults⁴⁰ and this has been associated with increased adiposity.^{41,42} The findings from animal studies corroborate the association of BPA exposure with adiposity. Specifically, studies show that it is lower (500 µg/kg/day) rather than higher dose (>5,000 µg/kg/day) of maternal BPA that is effective in promoting offspring weight gain.^{4, 5} Sex-specific effects are seen with some studies demonstrating increased postnatal growth in both males and females at maternal doses between 2.4–500 µg/kg/day,^{2, 43–45} only in males at maternal dose of 500 µg/kg/day⁵ and only in females at maternal dose of 100 µg/kg/day.⁴ The reported effective maternal BPA dose of 500 µg/kg/day is compatible with our *in vivo* studies where effects are seen only in the male offspring and consistent with study by Bartolomei et al.⁶ Further regardless of differing metabolism of BPA in rodents and humans,⁴⁶ pharmacokinetic studies indicate that BPA exposure of approximately 400–500 µg/kg/day yield blood concentrations of the unconjugated, bioactive form of BPA that is similar to that reported in human blood.⁴⁷

Previous studies on mice and rats indicate that endocrine disruptors^{48,49} and estrogen⁴⁹ have species and strain-dependent as well as organ-specific effects.⁴⁹ However, the overall sensitivity of various biological endpoints did not have pronounced differences between strains of rats.⁴⁹ With regard to adipogenesis, studies show that the two commonly used strains, Sprague Dawley and Wistar rats demonstrate increased adiposity in response to BPA exposure.^{50–53} Specifically, pregnant Sprague-Dawley exposed to BPA cause increased adipogenesis⁵⁰, metabolic perturbations⁵⁴ and induce epigenetic transgenerational inheritance of obesity.⁵⁵

Interestingly unlike in the offspring, increased plasma BPA levels did not adversely impact maternal body weight and body fat. This may be a result of continued exposure of developing fetus to BPA as stated above. There is also an implication that α-fetoprotein, which is secreted in high levels during development, may be involved. Normally, α-fetoprotein binds to estrogen and protects the developing fetus from estrogen effects. However, binding properties of some endocrine disruptors to α-fetoprotein may reduce this protection and expose the fetus to estrogen effects.^{56–59}

The present study demonstrates that despite normal birth weight, male newborns exposed to maternal BPA have a markedly increased postnatal growth rate and fat mass accumulation and hypertension. This elevated adipogenic potential is consistent with increased adipogenic (PPARγ, C/EBPα) and lipogenic (SREBP1) factors, which likely contribute to the hypertrophic adipocytes seen in BPA offspring. The underlying mechanism may be attributed to BPA-induced increased preadipocyte proliferation as demonstrated by *in vitro* BPA exposure studies. Specifically, preadipocytes exposed to *in vitro* BPA exhibit increased cell proliferation and increased Pref-1, suggesting an increased pool of committed preadipocyte stem cells for potential adipocyte differentiation.⁶⁰ Pref-1 is known to suppress adipocyte differentiation by induction of SOX9.^{60–62} In our studies of *in vitro* BPA

exposure, the failure of Pref-1 to induce SOX9, as evident by suppression of SOX9, indicates dysregulation of Pref-1/SOX9 that may facilitate adipocyte differentiation under appropriate stimuli (e.g., increased insulin/glucocorticoid),⁶³ high carbohydrate or high fat diet.^{64–66} Indeed, when preadipocytes are allowed to differentiate in presence of BPA, there is increased expression of adipogenic and lipogenic transcription factors together with more lipid-filled adipocytes. Collectively, this results in an inflammatory response as evident by increased expression of macrophage marker (CD68) and proinflammatory cytokine (TNF α). These changes are similar to the effects seen with *in vivo* maternal BPA exposure and consistent with previous reported *ex vivo* studies of 3T3-L1 pre-adipocyte cell lines,^{19,20,67–69} as well as murine and human^{70,71} primary stem cells. Furthermore in the present study, the early increased PPAR γ and later increased C/EBP α and SREBP1 suggest specific influence of BPA during perinatal and postnatal exposures. It is known that although PPAR γ and C/EBP α positively regulate each other's expression and cooperate to promote adipogenesis,⁷² C/EBP α is required for lipogenesis.⁷³

The mechanism underlying BPA-induced enhanced adipocyte proliferation and differentiation may involve epigenetic modifications via DNA and/or histone methylation,^{74,75} particularly of genes such as PPAR γ (Fujiki et al. 2009) and C/EBP α (Li et al. 2013). Methylase and demethylase enzymes that have been implicated in determining stem cell proliferation (self-renewal) and differentiation include DNMT (DNA methyltransferase) and LSD1 (lysine (K)-specific histone demethylase).⁷⁶ For example, treatment of 3T3-L1 preadipocytes with an inhibitor of DNA methylation (5'-aza-cytidine) or knockdown of LSD1 decreased proliferation and adipocyte differentiation, resulting in downregulation of PPAR γ .^{77–79} This is consistent with our findings on *in vitro* BPA exposure, that is, increased preadipocyte proliferation with increased protein expression of DNMT3a. The lack of dose response seen in the case of C/EBP α is presently unknown. However, it may involve BPA or direct transcription factor-mediated chromatin interaction and accessibility.^{74, 80} While the current data suggest BPA alters transcriptional regulation of genes involved in proliferation, the role of DNA methylation in preadipocytes remains to be established and further studies of site-specific epigenetic modification of specific genes are required to fully elucidate BPA-induced changes in adipogenesis.

Although the sex-specific effects of BPA are well-documented including the differential susceptibility of males and females to different doses of BPA^{47,81–85} the underlying mechanism remains unclear.⁸² The plausible explanation may involve sex-hormones, genomic and non-genomic pathway involving nuclear estrogen receptors, differing developmental pattern and/or epigenetic influence.^{47,83,86}

CONCLUSION

Our data (Table 2) confirm that primary adipose progenitor cells are vulnerable to endocrine disruption by BPA resulting in altered proliferation and differentiation in early life independent of systemic influences. Enhanced adipose proliferation and differentiation indicate the potential for maternal/fetal BPA exposure to program an increased risk of offspring obesity and consequent metabolic abnormalities.^{4, 18, 87, 88}

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Figure 1A: Effects of Maternal BPA Exposure on Maternal Body Weights, Food Intake and Water Intake Maternal daily body weights and water intake during pregnancy and lactation of BPA (□) and Control (■) dams. Values are means $\pm$ SE of n=5 litters. Figure 1B: Effects of Maternal BPA Exposure on Maternal Body Fat
Maternal body fat and retroperitoneal adipocyte cell size and images (scale bar = 100μm) at end of lactation of BPA (□) and Control (■) dams. Values are means $\pm$ SE of n=5 litters.

Figure 2: Maternal BPA Effects on Offspring Body Weights

Body weights of 1 day old newborns, and body weights of 3 and 24 weeks old male and female offspring. Values are means $\pm$ SE of n=5 from 5 litters per group; * p<0.05 BPA (□) vs. Control (■).

Figure 3: Maternal BPA Effects on Offspring Adiposity

Percentage body fat of 3 and 24 weeks old male and female offspring. Values are means $\pm$ SE of n=5 from 5 litters per group; * p<0.05 BPA (□) vs. Control (■).

Figure 4: Maternal BPA Effects on Offspring Blood Pressure

Systolic blood pressure of 6 weeks old male and female offspring. Values are means $\pm$ SE of n=5 from 5 litters per group; * p<0.05 BPA (□) vs. Control (■).

Figure 5: Maternal BPA Effects on Offspring Adipogenic and Lipogenic Factors

(a) Inguinal adipose tissue protein expression of PPAR γ and C/EBP α in 1 day old male newborns, (b) Retroperitoneal adipose protein expression of PPAR γ , C/EBP α and SREBP1, and (c) retroperitoneal adipocyte cell size and images (scale bar = 100 μ m) in 3 week old male offspring. Values are means $\pm$ SE of n=5 of pooled adipose from each of 5 litters per group; * p<0.05 BPA ($\square$) vs. Control ($\blacksquare$).

Figure 6: Maternal BPA Effects on Adipose Tissue Inflammation in 3 Week Old Male Offspring
Retroperitoneal adipose protein expression of CD68 and TNF α in 3 week old male offspring. Values are means $\pm$ SE of n=5 of pooled adipose from each of 5 litters per group; * p<0.05 BPA (□) vs. Control (■).

Figure 7: In Vitro BPA Effects on Preadipocytes

Inguinal adipose tissue from 1 day old Control newborns were cultured in DMEM (undifferentiated) media and treated with DMSO (Control) or BPA (0, 1, 10, 20 μ M) for 5 days. (a) Preadipocyte image, (b) proliferative index measured at 520 OD and (c) protein expression of Pref1 and Sox9 (results are shown for two doses due to loss of 20 μ M BPA protein extract) with representative blot shown. Values are fold change (mean $\pm$ SE) of n=4 of pooled adipose from each of 4 litters; * P< 0.05 BPA ($\square$) vs. Control ($\blacksquare$).

Figure 8: In Vitro BPA Effects on Differentiated Adipocytes

Inguinal adipose tissue from 1 day old Control newborns were cultured in DMEM media and preadipocytes were allowed to differentiate in presence of BPA for 5 days. (a) Protein expression of PPAR γ and C/EBP α with representative blot shown. (b) Adipocytes were fixed with 4% paraformaldehyde and stained for lipid (Oil Red O; red) and nucleus (DAPI; blue). Scale bar = 100 μ m. Protein expression of SREBP1 with representative blot shown and lipid content. Values are fold change (mean $\pm$ SE) of n=4 of pooled adipose from each of 4 litters; * P< 0.05 BPA ($\square$) vs. Control ($\blacksquare$).

Figure 9: In Vitro BPA Effects on Epigenetic Factors

Inguinal preadipocytes from 1 day old Control newborns were cultured in DMEM media with BPA (1, 10, 20 μM) for 5 days. Protein expression of DNMT3a and LSD1 with representative blot shown. Values are fold change (mean±SE) of pooled cells n=4 from each of 4 litters; * P<0.05 BPA (□) vs. Control (■).

Table 1:**In Vivo Maternal BPA Exposure: Drinking Water**

	Control N=5 Litters	BPA N=5 Litters	Parameters Measured
Female Non-Pregnant Age: 9-12 Weeks	BPA-free	BPA	Body weights, Water Intake
Mating Age: 12 Weeks	BPA-free	BPA	Body weights, Water Intake, Tail Bleed
Pregnancy GA: 0-21 Days	BPA-free	BPA	Body weights, Water Intake
Newborns Age: 1 Day	BPA-free	BPA	Body Weights, Blood, Adipose Tissue
Lactation Nursing: 0-21 Days	BPA-free	BPA	Body weights, Water Intake, Blood, DEXA
Offspring Age: 3 Weeks	BPA-free	BPA-free	Body Weights, DEXA, Adipose Tissue
Offspring Age: 6 Weeks	BPA-free	BPA-free	Body Weight, Blood Pressure
Offspring Age: 24 Weeks	BPA-free	BPA-free	Body Weight, DEXA

In Vivo Maternal BPA Exposure: Non-pregnant female rats at 9 weeks of age were allowed drinking water that was BPA-free (Control group) or contained BPA (BPA group). At 12 weeks of age, tail blood was obtained for BPA analysis and all females were mated and continued on same drinking water regimen throughout pregnancy and lactation. Parameters measured at various time points are indicated. At each offspring ages, N=5 males were studied from 5 separate litters.

Table 2:Summary Data of *In Vivo* Exposure to Maternal BPA

Parameters	Ages			
	1 Day	3 Weeks	6 Weeks	24 Weeks
Body Weight	↔	↑	↑	↑
Body Fat	-	↑	-	↑
Blood Pressure	-	-	↑	-
Adipose Tissue				
Size	-	↑	-	-
PPAR γ	↑	↔	-	-
C/EBP α	↔	↑	-	-
SREBP1	↔	↑	-	-
CD68	-	↑	-	-
TNF α	-	↑	-	-

Changes at various ages are summarized for male offspring exposed to maternal BPA. Increased (↑); no change (↔); not studied (-).